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ORIGINAL ARTICLE

Enantiomeric separation of a melatonin agonist Ramelteon using amylose-based chiral stationary phase

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Abstract A new simple isocratic chiral liquid chromatographic method was developed for the enantiomeric purity of Ramelteon [(*S*)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-b]furan-8-yl) ethyl]-propionamide], a melatonin agonist in bulk drugs. The chromatographic separation was achieved on Chiralpak AD-H, 250 mm × 4.6 mm, 5 μm column using a mobile phase system consisting of *n*-hexane, ethanol and methanesulfonic acid in the ratio of 900:100:0.1 (v/v/v). The mobile phase was pumped on the column at the flow rate of 1 mL min^{-1}. Addition of methane sulfonic acid in the mobile phase enhanced chromatographic efficiency and resolution between the enantiomers. The resolution between the enantiomers was found to be more than four. The developed method was subsequently validated and proved to be accurate and precise. The experimentally established limit of detection and quantification of (*R*)-enantiomer were found to be 25.5 and 77.2 ng ml^{-1}, respectively, for 20 μl injection volumes. The percentage recovery of (*R*)-enantiomer was ranged from 98.5 to 101.9 in bulk drug samples of Ramelteon. The stability of Ramelteon sample in analytical solution was checked for about 48 h at room temperature and was found to be stable for about 48 h. The proposed method was found to be suitable and accurate for the quantitative determination of (*R*)-enantiomer in drug substance.

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1. Introduction

The biological activity of chiral substances often depends upon their stereochemistry. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics (Stinson, 2000, 2001; Thayer, 2008). Analysis of the enantiomeric purity of chiral drug candidates has become very important particularly in the pharmaceutical and biological fields, because few enantiomers of racemic drugs have relatively different pharmacokinetic

properties and diverse pharmacological or toxicological effects (FDA, 1992; Ariens, 1984, 1986, 1987).

Enantiomeric separations have acquired importance in all stages of drug development and commercialization process. Therefore, the development of new methods for efficient chiral separations mainly based on HPLC (Nikolic et al., 2009; Kubota et al., 2010; Kasawar and Farooqui, 2009; Meng et al., 2010), capillary electrophoresis (CE) (Van Eeckhaut and Michotte, 2006; Halabi et al., 2004) or gas chromatography (GC) (Schurig, 2002; Ramos Mda et al., 2003) is more than necessary. The chromatographic separation of enantiomers using high-performance liquid chromatography (HPLC) with chiral stationary phases (CSPs) is one of the most useful and popular techniques for enantiopurity analysis in pharmaceutical preparations and biological fluids (Toribio et al., 2006; Simplicio et al., 2006; Sellers et al., 2006).

Insomnia is a common sleep disorder with significant potential for deleterious effects on activity of daily living, productivity and overall quality of life. Ramelteon, a highly selective agonist for melatonin subtypes 1 and 2 receptors, is a hypnotic agent approved by the US Food and Drug Administration (FDA) for the treatment of insomnia characterized by difficulty falling asleep (Borja and Daniel, 2006).

Rameleteon, (*S*)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno-[5,4-*b*]furan-8-yl)ethyl] propionamide (Fig. 1) structure is composed of a substituted tetrahydroindenofuran derivative containing a propionamide moiety with one chiral center, and the compound is produced as the (*S*) enantiomer (Yamano et al., 2006).

A thorough literature survey revealed that none of the most recognized pharmacopoeias or journals include this drug for the determination of chiral purity. The chiral purity of drug is very important since only *S*-isomer of it is active and *R*-isomer is inactive. So it is felt essential to develop a liquid chromatographic procedure which will serve as a reliable, accurate, sensitive and stability indicating normal phase HPLC method for the determination of chiral purity of Ramelteon.

2. Experimental

2.1. Chemicals

Ramelteon and (*R*)-enantiomer were kindly supplied by School of chemical sciences, S.R.T.M. University Nanded, India and the chemical structure is shown in Fig. 1. HPLC grade *n*-hexane and ethanol were purchased from Merck Ltd., Germany. Laboratory reagent grade diethyl amine, trifluoroacetic acid and methanesulfonic acid were purchased from Merck Ltd., Germany.

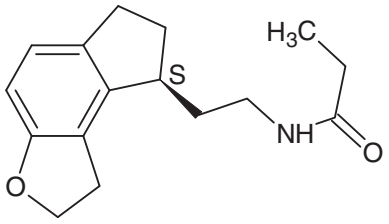

Figure 1 Molecular structure of (*S*)-Ramelteon.

2.2. Equipment

A Waters 2695 separation module (USA) equipped with an auto injector was utilized for method development and validation. Empower software was used for data acquisition and system suitability calculations.

2.3. Sample preparation

A solution containing racemic mixture of Ramelteon (0.1 mg mL^{-1}) and sample solution of Ramelteon (1 mg mL^{-1}) was prepared in *n*-hexane and ethanol in the ratio of 60:40 (v/v).

2.4. Chromatographic conditions

The chromatographic conditions were optimized using an amylose-based chiral stationary phase Chiralpak AD-H 250 mm × 4.6 mm, 5 μm (Daicel), with safeguarded column of 1 cm long. The mobile phase consisting of *n*-hexane, ethanol and methanesulfonic acid in the ratio of 900:100:0.1 (v/v/v) was pumped through the column at the flow rate of 1.0 mL min^{-1}. The column compartment was maintained at 25 °C and the detection was carried out at a wavelength of 220 nm. The injection volume was 20 μl. Amylose-based chiral stationary phase Chiralpak AS-H (ChromTech), Cellulose based chiral stationary phase Chiralcel OD-H (Diacel) and pirkle based chiral stationary phase Whelk-O-1 (Merck) were also employed during method development.

2.5. Validation of the method

2.5.1. Specificity

The specificity of the developed LC method was determined in the presence of its related impurities and degradation products. Forced degradation studies were also performed on Ramelteon bulk drug sample to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study include light (1.2 million lux h/m^2/22 h), heat (105 °C/24 h), humidity (40 °C/75% RH/7 days), acid hydrolysis (0.1 N ethanolic HCl/5 ml/3 h reflux), base hydrolysis (0.1 N ethanolic NaOH/5 ml/3 h reflux), and peroxide degradation (3% w/v H_2O_2/5 ml/3 h reflux). About 5–15% degradation was observed in forced degradation studies and (*R*) enantiomer observed was about 0.3% in both stressed and unstressed samples. The typical chromatograms of control sample and stressed samples were represented in Fig. 2. Peak purity of stressed sample of *S*-Ramelteon was checked by using photo diode array detector. Purity angle was found less than purity threshold in all stress samples and no interference was found from the blank which demonstrate the analyte peak homogeneity.

2.5.2. Method reproducibility

Method reproducibility was determined by measuring repeatability and intermediate precision (between-day precision) of retention times and peak areas for each enantiomer. In order to determine the repeatability of the method, replicate injections (n = 6) of a 1.0 mg/ml solution containing Ramelteon and (*R*)-enantiomer was carried out. The intermediate precision was also evaluated over 3 days by performing six successive injections each day.

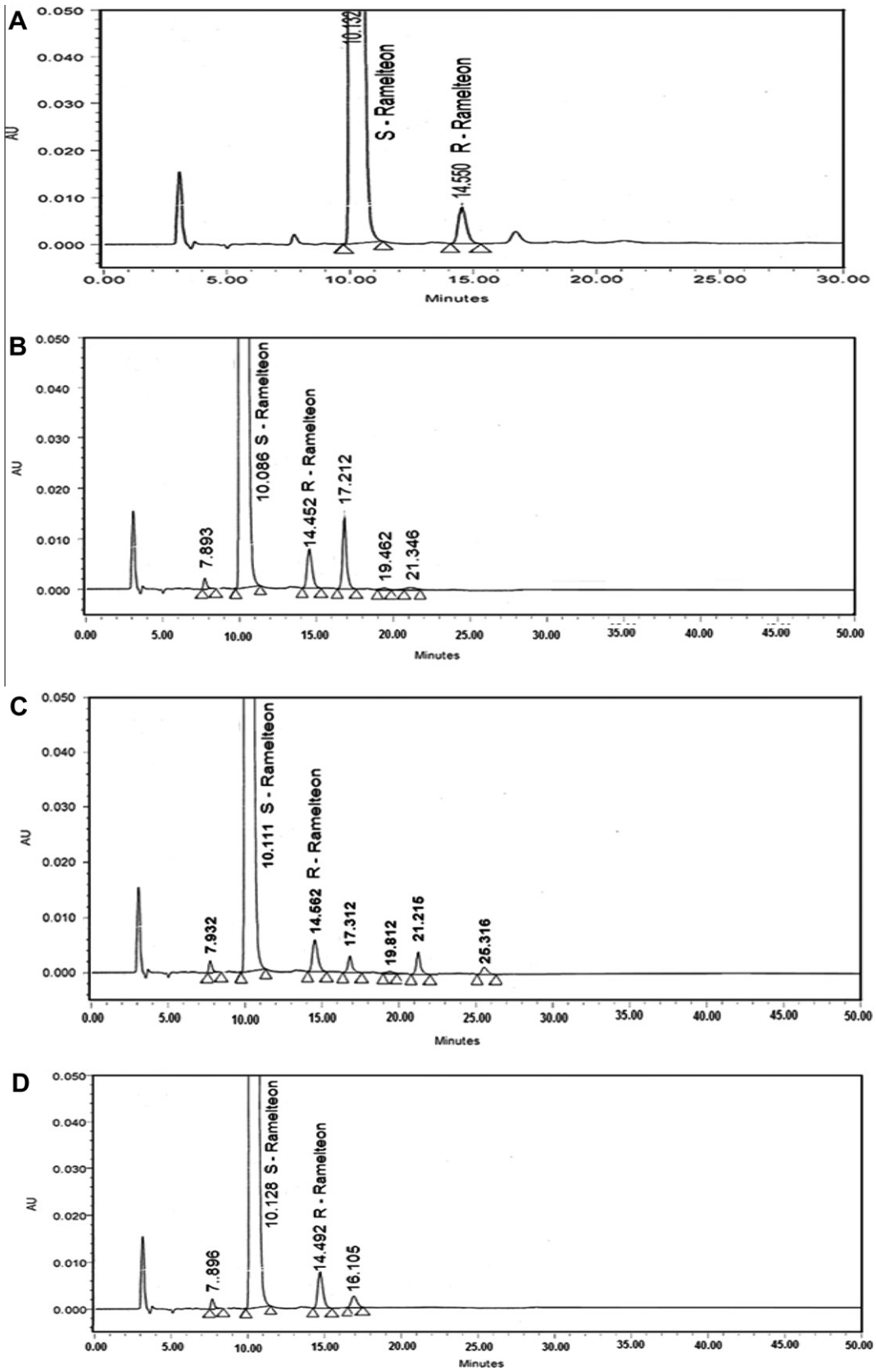

Figure 2 Typical HPLC chromatograms of pure and stressed samples. (A) Untreated sample, (B) sample stressed under heat, (C) sample stressed under UV, (D) sample stressed under humidity, (E) sample treated with acid, (F) sample treated with base, and (G) sample treated with peroxide. (Column: Chiralpak AD-H 250 mm × 4.6 mm, 5 μm, mobile phase: *n*-hexane:ethanol:methanesulfonic acid (900:100:0.1, v/v/v), flow rate: 1 ml/min, UV detection: 220 nm).

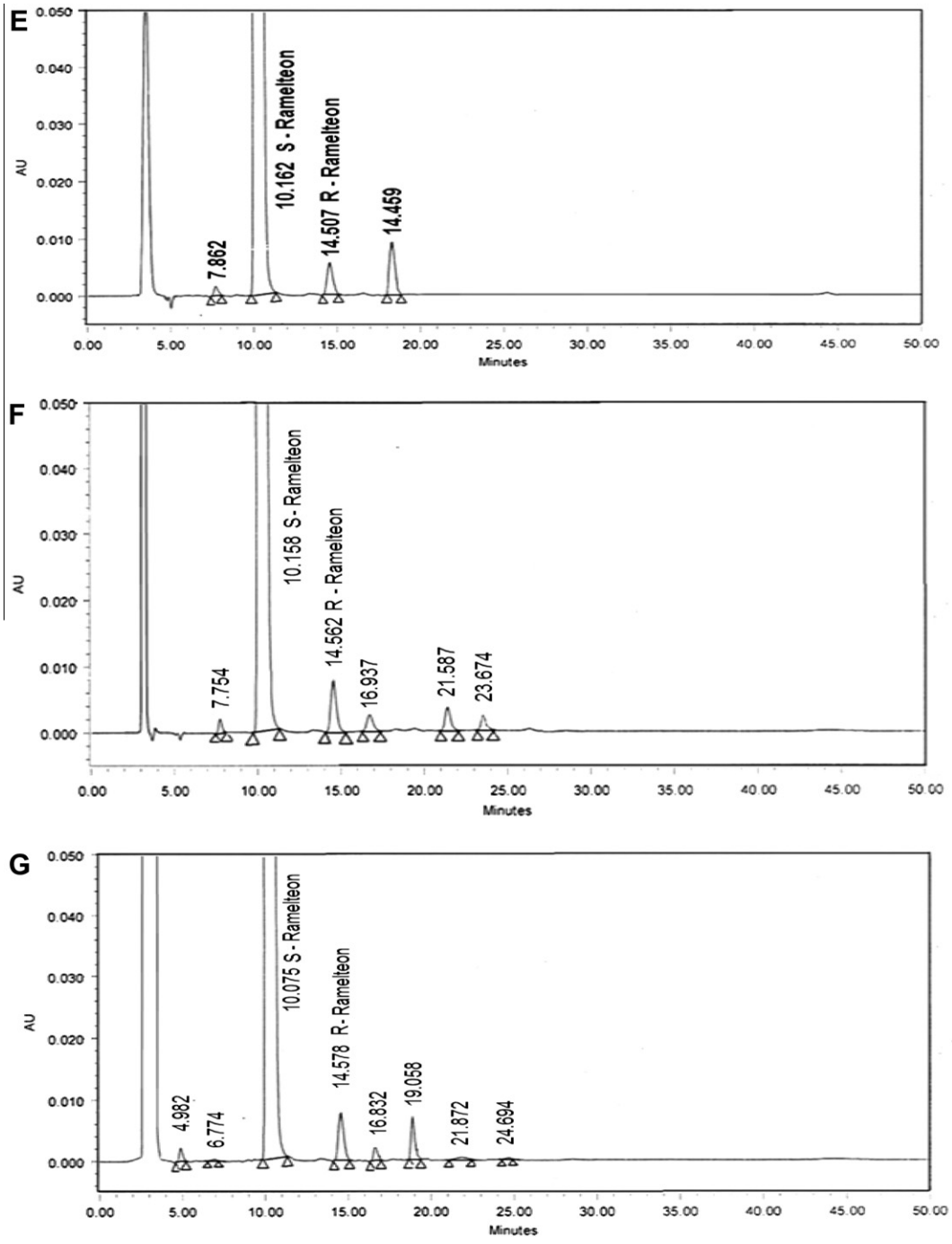

Figure 2 (*continued*)

2.5.3. Linearity of (R)-enantiomer

The linearity of detector response was assessed by preparing calibration curve of sample solutions (*R*-enantiomer) of 14 concentration levels covering from 20 ng mL^{-1} to 2400 ng mL^{-1} in *n*-hexane and ethanol in the ratio of 60:40 (v/v). Regression curve was obtained by plotting peak area versus respective concentration, using the least squares method. The slope and intercept of the calibration curve was calculated.

2.5.4. Limit of detection and limit of quantification of (R)-enantiomer

The limit of detection is defined as the lowest concentration of analyte that can be clearly detected above the baseline signal, while the limit of quantification is defined as the lowest concentration of analyte that can be quantified with suitable precision and accuracy. The linearity performed above is used for the determination of limit of quantification and detection. Residual standard deviation (σ) method was applied to predict the

LOQ and LOD values using the following formula (a), (b) and the precision was established at these predicted levels. The results are tabulated in Table 2

$$LOQ = 10\sigma/S \quad (a)$$

$$LOD = 3.3\sigma/S \quad (b)$$

where σ = residual standard deviation of response; S = slope of the calibration curve

2.5.5. Quantification of (R)-enantiomer in bulk sample

Standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantification of (*R*)-enantiomer in bulk drug samples. The study was carried out in triplicate at 0.1%, 0.2% and 0.24% of the target analyte concentration.

2.5.6. Robustness

Robustness of the method was performed by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 to 0.9 mL min^{-1} and 1.1 mL min^{-1}. The ethanol strength of mobile phase was varied by ±2% while column temperature was varied by ±5 °C. The solution of racemic mixture was injected in each varied conditions and the resolution between the isomers was checked. In all deliberately varied conditions, the resolution between the isomers was found to be well within the acceptance limit of 2% indicating the method robustness.

2.5.7. Solution stability and mobile phase stability

Stability of Ramelteon in analytical solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 2 days. Content of (*R*)-enantiomer was checked for 6 h interval up to the study period. Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in Ramelteon sample solutions prepared freshly at 6 h interval for 2 days. Same mobile phase was used during the study period.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The aim of this work is to separate the enantiomers for the accurate quantification of (*R*)-enantiomer. Racemic mixture solution of 10 mg mL^{-1} prepared in *n*-hexane and ethanol in the ratio of 60:40 (v/v) was used in the method development. To develop a rugged and suitable LC method for the separation of Ramelteon enantiomers, different mobile phases and stationary phases were employed. To achieve the chromatographic separation, various chiral columns like Chiralcel ODH, Chirapak AS-H, Chiralpak AD-H (Diacel) and Whelk-O-1 (Merck) were used. Various experiments were conducted, to select the best stationary and mobile phases that would give optimum resolution and selectivity for the enantiomers. Poor separation was found on Chiralcel OD-H, Whelk-O-1 and Chiralpak AS-H columns using different possible mobile phases. There is an indication of separation on Chiralpak AD-H column using a mobile phase consisting of *n*-hexane, ethanol, and diethylamine in the ratio of 800:200:1 (v/v/v) as well as *n*-hexane, ethanol, trifluoroacetic acid in the ratio of 800:200:1 (v/v/v) but the peak shapes were found to be broad. Introduction of methanesulfonic acid in the mobile phase improved the chromatographic efficiency and resolution between the enantiomers. Very good separation was achieved on Chiralpak AD-H column (resolution between enantiomers was found greater than seven) using the mobile phase system *n*-hexane, ethanol, methanesulfonic acid in the ratio of 900:100:0.1 (v/v/v) (Fig 3). Reversal of the elution order of the enantiomers on changing from ethanol to 1-propanol was observed on Chiralpak AD-H column. The chiral discrimination of enantiomers occurs when they bind with the stationary phase forming transient diastereomeric complexes. The most important interactions between the analyte and the chiral stationary phase (CPS) are hydrogen bonding, dipole–dipole interactions, and π–π interactions, together with the rigid structure (cellulose-based CSP) or helical structure (amylose-based CSP) of the chiral polymer bound to the support. The enantioselectivity changes suggest that the ethanol affects the steric environment of the chiral cavities or channels of the stationary phase. Ramelteon has only one chiral center and an amylose-based chiral stationary phase has five chiral centers per unit. It is presumed that it could be due to high probability of interaction, better resolution was found on Chiralpak AD-H column. Due to the better chromatographic results obtained on the Chiralpak AD-H column, the method validation was carried out on the same column. In the optimized method, the typical retention times of Ramelteon and (*R*)-enantiomer were about 10 and 14.4 min, respectively. The enantiomeric separation of

Table 1 System-suitability report.

Compound (n = 3)	Rt	Rs	N	T
Ramelteon	10.2	–	6717	1.51
R(−) enantiomer	14.4	7.2	7544	1.51

n = 3 determinations. Rt: retention time, Rs: USP resolution, N: number of theoretical plates (USP tangent method), T: USP tailing factor.

Table 2 Validation results of the developed chiral LC method.

Validation parameter	Results
Repeatability (n = 6, %RSD)	
Retention time (*S*-enantiomer)	0.2
Retention time (*R*-enantiomer)	0.1
Peak area (*S*-enantiomer)	0.3
Peak area (*R*-enantiomer)	0.7
Intermediate precision (n = 18, %RSD)	
Retention time (*S*-enantiomer)	0.3
Retention time (*R*-enantiomer)	0.2
Peak area (*S*-enantiomer)	0.4
Peak area (*R*-enantiomer)	0.8
LOD–LOQ (R-enantiomer)	
Limit of detection (ng/ml)	25.5
Limit of quantification (ng/ml)	77.2
Precision at LOQ (%RSD)	3.3
Linearity (R-enantiomer)	
Calibration range (ng mL^{-1})	80–2400
Calibration points	12
Correlation coefficient	0.9997

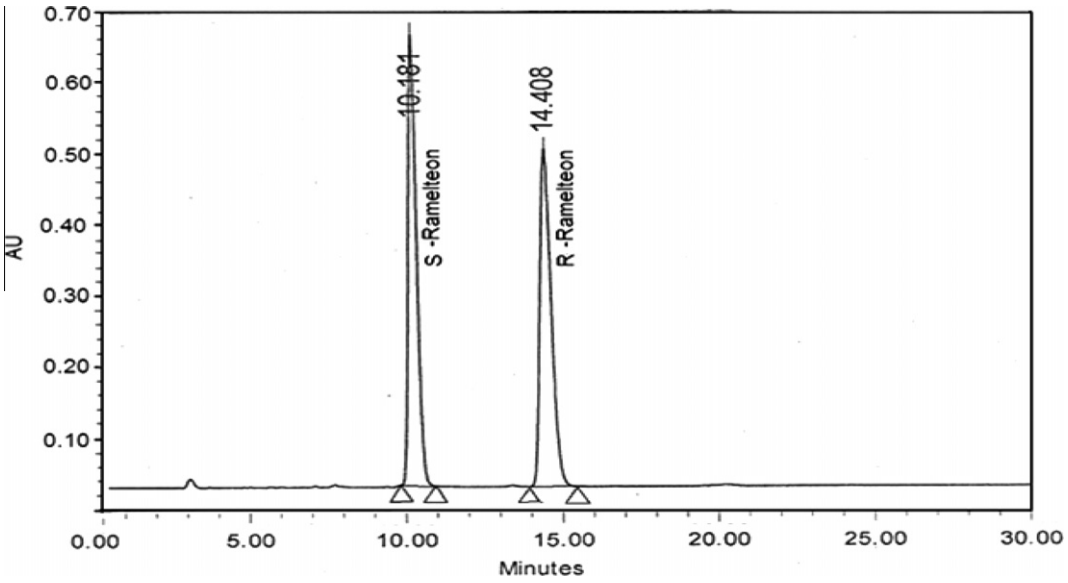

Figure 3 Enantiomeric resolution of Ramelteon on Chiralpack AD-H column. (Column: Chiralpak AD-H 250 mm × 4.6 mm, 5 μm, mobile phase: *n*-hexane:ethanol:methanesulfonic acid (900:100:0.1, v/v/v), flow rate: 1 ml/min, UV detection: 220 nm).

Table 3 Recovery results of (*R*)-enantiomer in bulk drugs.

Amount added (μg)	Amount recovered (μg)	Recovery (%)	RSD (%) $n = 3$
51.3	51.7	100.8	1.2
102.5	102.8	100.3	1.4
123.0	122.5	99.6	1.6

RSD: relative standard deviation.

Table 4 Robustness of the chiral LC method.

Parameter	USP resolution between Ramelteon and (*R*)-enantiomer
Flow rate (ml/min)	
0.9	7.6
1.0	7.2
1.1	6.9
Column temperature (°C)	
20	7.5
25	7.2
30	7.0
Ethanol percentage in mobile phase	
8	7.4
10	7.2
12	7.1

Ramelteon on Chiralpak AD-H column was shown in Fig. 3. The system suitability test results of the chiral LC method on Chiralpak AD are presented in Table 1.

3.2. Validation results of the method

In the repeatability study, the percentage relative standard deviation (%RSD) was found less than 0.5 for the retention times of both the enantiomers, 0.7 for (*R*)-enantiomer peak area and 0.3 for (*S*)-enantiomer peak area. In the intermediate precision study, results show that %RSD values were in the same order of magnitude than those obtained for repeatability. The limit of detection (LOD) and limit of quantification (LOQ) concentrations were estimated to be 25.5 and 77.2 ng mL^{-1} for (*R*)-enantiomer. Good linearity was observed for (*R*)-enantiomer over the concentration range of 80–2400 ng mL^{-1}, with the linear regression equation $y = 31870.908x + 326.919$ (correlation coefficient $R = 0.99968$). The results are tabulated in Table 2. The standard addition and recovery experiments were conducted for (*R*)-enantiomer in bulk samples in triplicate at 0.1%, 0.2% and 0.24% of analyte concentration and percentage recovery was ranged from 98.5 to 101.9 (Table 3).

The chromatographic resolution between Ramelteon and (*R*)-enantiomer peaks was used to evaluate the method robustness under modified conditions. The resolution between Ramelteon and (*R*)-enantiomer was greater than 6.0, under all conditions tested (Table 4), demonstrating sufficient robustness. No significant change in the (*R*)-enantiomer content was observed in Ramelteon sample during solution stability and mobile phase stability experiments. Hence, Ramelteon sample solution and mobile phase was found stable for about 48 h.

4. Conclusion

A simple, rapid and accurate normal phase chiral LC method was described for the enantiomeric separation of Ramelteon. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the quantitative determination of chiral impurity ((*R*)-enantiomer) in bulk materials.

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